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Characterization of the infant gut microbiota in a cohort of 330 Danish children at 9, 18 and 36 months by quantitative PCR array (GULDA) analysis

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Introduction

We have developed a qPCR-based array (GUt Low Density Array, GULDA), which simultaneously determine the relative abundance of >30 different bacterial 16S rRNA gene targets in a given DNA-sample covering selected phylogenetic levels (Bergström *et al*., FEMS Microbiology Letters 337 (1), 38-47, **2012**). GULDA was applied to fecal DNA from 330 healthy Danish infants, sampled at 9, 18 and 36 months after birth, enabling characterization of interbacterial relationships by multivariate data analysis (Principal Component Analysis), univariate data analysis (ANOVA, t-tests) and non-parametric pairwise Spearman correlations. Interpretation of these patterns in relation to previously determined nutritional, anthropometrical (growth indices), and blood sampled parameters was used to increase understanding of gut microbial physiology. Particular emphasis was given to possible early life microbiota biomarkers of obesity, given the correlation of early life overweight with adult obesity and related life-style diseases. Few studies have undertaken similar longitudinal and multiparametric analysis for such numerous participants. The concomitant measures of bacteria from all relevant phyla on multiple taxonomic levels give a unique possibility for recognition of gut bacteria clusters. Due to study dropout, non-compliance and failed fecal DNA purifications, at total of 658 fecal samples were available for the analysis (>200 in each of the three age groups).
With exception of breastfeeding and certain obesity indices (Figures 6 and 7), we found quite few consistent correlations between the gut bacteria and the physiological parameters, hence the primary focus of the current presentation is on the interbacterial correlations.

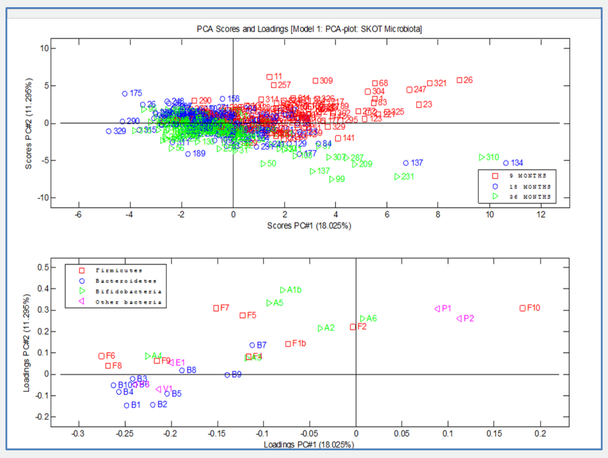

Figure 1 – Principal component analysis (PCA) of the GULDA microbiota. Upper plot=Individuals (Scores); Lower plot=Bacteria (Loadings). This figure shows the two primary principal components, PC1 and PC2, which explain approximately 30% of data variation. Only individuals who completed all three fecal samplings were included, giving a total of 396 samples from 132 individuals. There is a strong temporal development moving along PC1 from left to right, moving from 9 to 36 months, and a moderate temporal development, moving along PC2, from top and down. Higher diversity of 9 months samples with corresponding fewer bacterial species relative to 18 and 36 months is evident. Bacteria codes (see Figure 3).

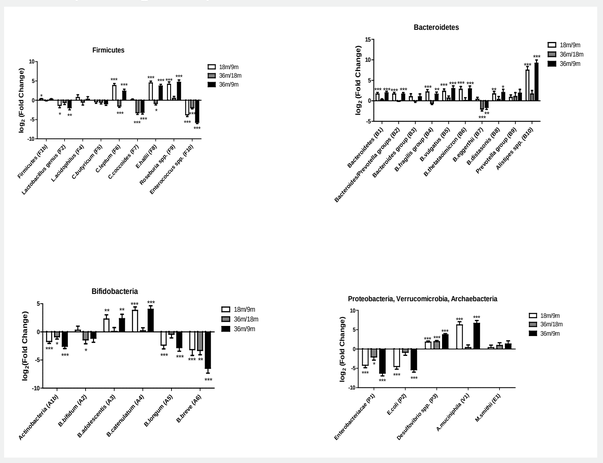

Figure 2: Temporal development of the gut microbial composition
log2 (Fold changes) of all GULDA bacteria from 9 to 18 months, 18 to 36 months, and 9 to 36 months. Overall the majority of changes in the gut composition take place between 9 and 18 months with less change occurring between 18 and 36 months. Specific increases were seen for most of the targeted Bacteroidetes, C.leptum, E.hallii, Roseburia spp., Desulfovibrio spp. and A.muciniphila, while decreases were observed for Lactobacillus genus, Enterococcus spp., Actinobacteria , Enterobacteriacae and E.coli. A few GULDA targets showed opposing changes compared to the temporal development of the respective phylum target.

Statistical significance of one-sided t-tests.

*p<0.05, **p<0.01, ***p<0.001

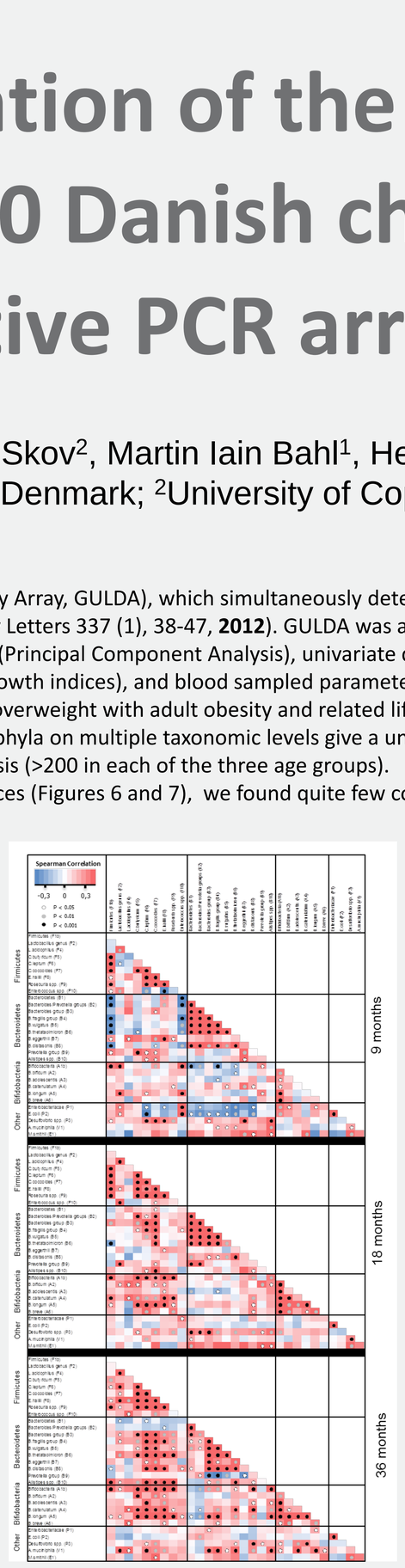

Figure 3: Spearman pairwise correlation map of all interbacterial correlations at 9, 18, and 36 months sampling.
Not surprisingly, many positive correlations of targets belonging to the same phylum were observed.

At 9 months most members of the *Bacteroidetes* phylum (B1-B6, B8) were negatively correlated to the *Firmicutes* phylum, including *Enterococcus* spp., however such correlations were not observed at neither 18 nor 36 months. Indeed at both 18 and 36 months significant positive correlations were found between several bacterial groups within the *Firmicutes*, including *C. butyricum*, the *C. leptum* group and the *C. coccoides* group and members of the *Bacteroidetes* including *Bacteroides* spp. and *Alistipes* spp. This development may reflect dietary changes as breastfeeding is weaned off and normal Western adult diet, rich in long-chained carbohydrates and animal fats and protein, is introduced between 9 and 18 months.

Interestingly, *Prevotella spp*. showed negative correlation to specific *Bacteroides* targets at 36 months, possibly indicating the earliest observed signs of enterotype stratification.
Since each time point was analyzed separately, the included number of individuals was >200 in each group.

P values and corresponding False Discovery Rates (FDR):
9 months (p<0.05; FDR=0.17, p<0.01, FDR=0.05, p<0.001; FDR=0.01)
18 months (p<0.05; FDR=0.17, p<0.01, FDR=0.04, p<0.001; FDR=0.01)
36 months (p<0.05; FDR=0.12, p<0.01, FDR=0.04, p<0.001; FDR=0.005)

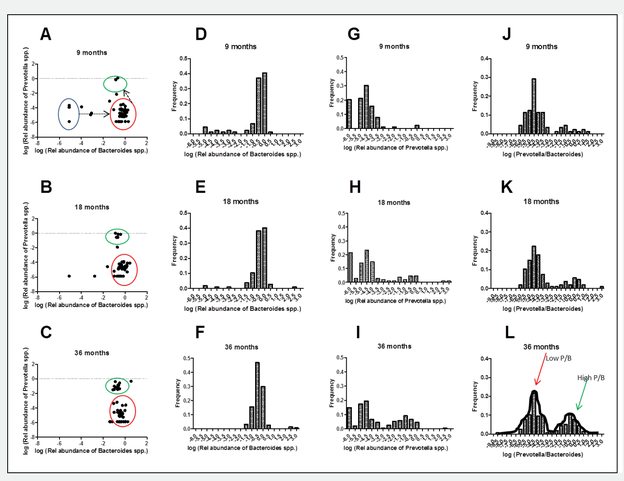

Figure 4: Enterotype development from 9 to 36 months.

Relative abundances of Bacteroides and Prevotella targets show a distinct development from 9 to 36 months (A-C), arguably moving from trace amounts of either bacteria just after birth (blue circle) to a Bacteroides dominated microbiota (red circle) at 9 months. From 9 to 36 months, an increasing, yet smaller subgroup of Prevotella dominated individuals appear (green circle), indicating segregation of specific individuals from a Bacteroides dominated into a Prevotella dominated enterotype. These findings were paralleled by the temporal developments of frequency distributions of Bacteroides (D-F) and Prevotella (G-I) and particularly of the P/B-ratio from 9 to 36 months. A distinct bimodal P/B-pattern seems to be established from 9 to 36 months (J-L), clearly driven by stratification into individuals with either high (green) or low (red) relative Prevotella abundances. Possible causes of this segregation could be genetic or dietary, but could not be clarified in the present study. Since valid qPCR results for both primer sets were required at all time points, the number of individuals was lower than in Figures 1 and 2.

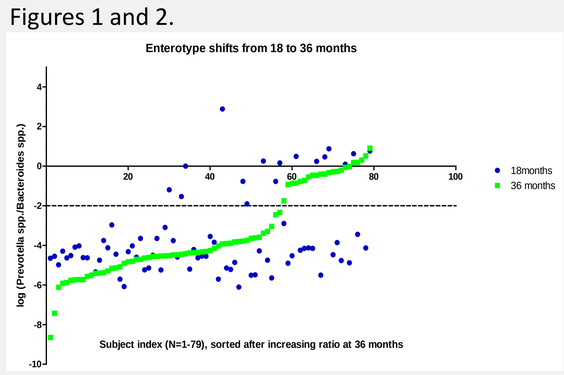

Figure 5: Enterotype shifts from 18 to 36 months

Examination of individuals giving valid qPCR results for both 18 and 36 months samplings, illustrated that: 48/79 were in the low P/B group, while 8/79 were in the high P/B at both time points. 14/79 and 9/79 shifted enterotype from low to high and high to low P/B, respectively. The separation into the two enterotypes is quite clear. Comparison of individuals shifting to the high P/B group with individuals staying in the lower P/B group showed a slight significant correlation to increase in BMI, but these results should be taken with precautions, given the large differences in group sizes.

Breastfed vs not breastfed at 9 months

	9 months	18 months	36 months
Firmicutes (F1b)	0,6554	0,1873	0,4915
Lactobacillus genus (F2)	0,0002***	0,9547	0,0821
L.acidophilus (F4)	0,7673	0,1582	0,6203
C.butyricum (F5)	0,3116	0,4549	0,4951
C.leptum (F6)	0,0216*	0,7294	0,6451
C.coccoides (F7)	0,0034**	0,0233*	0,7511
E.hallii (F8)	0,0233*	0,2389	0,6853
Roseburia spp. (F9)	0,0026**	0,4348	0,4603
Enterococcus spp. (F10)	0,0143*	0,166	0,4637
Bacteroidetes (B1)	0,0016**	0,1681	0,7768
Bacteroides/Prevotella groups (B2)	0,0027**	0,0439*	0,1101
Bacteroides group (B3)	0,008**	0,3063	0,9444
B.fragilis group (B4)	< 0,0001***	0,1739	0,8995
B.vulgatus (B5)	0,0022**	0,0435*	0,5356
B.thetataoimicron (B6)	0,0004***	0,0321	0,5817
B.eggerthii (B7)	0,2998	0,5847	0,7772
B.distasonis (B8)	0,0916	0,3863	0,8184
Prevotella group (B9)	0,3472	0,7174	0,0425*
Alistipes spp. (B10)	0,8796	0,4563	0,4115
Actinobacteria (A1b)	< 0,0001***	0,266	0,613
B.bifidum (A2)	0,2247	0,9662	0,9999
B.adolescentis (A3)	0,7229	0,4238	0,6833
B.catenulatum (A4)	0,7438	0,2498	0,7887
B.longum (A5)	0,054	0,7761	0,9433
B.breve (A6)	0,815	0,3147	0,0208*
Enterobacteriacae (P1)	0,2104	0,943	0,0496*
E.coli (P2)	0,0765	0,5992	0,2789
Desulfovibrio spp. (P3)	0,0125*	0,462	0,875
A.muciniphila (V1)	0,0037	0,4534	0,4889
M.smithii (E1)	0,1431	0,228	0,2695

Increased in breastfed at 9 months
Decreased in breastfed at 9 months
*p<0.05; **p<0.01**; ***p<0.001

Figure 6: Effect of breastfeeding on infant gut microbiota

Numbers denote p value of Mann-Whitney statistical test between the relative abundances of the GULDA bacteria at 9, 18 and 36 months, dependent on whether or not, the infants were still breastfed at the 9 months examination. Cessation of breastfeeding seems to be the major factor in maturing the gut microbiota, but the effect wanes off for most targets after 9 months.

	N	Fold change: 9 to 18 months VS. ΔBMI$_z$ (18m/9m) P	R	Fold change: 18 to 36 months VS. ΔBMI$_z$ (36m/18m) P	R	Fold change: 9 to 36 month VS. ΔBMI$_z$ (36m/9m) p	R
Firmicutes							
Firmicutes (F1b)	132	*0.02**	*0.20*	0.19	0.12	0.62	0.04
C.leptum (F6)	132	*0.02**	*0.21*	0.44	-0.07	0.05	0.17
E.hallii (F8)	131	*0.03**	*0.19*	0.73	-0.03	0.94	0.01
Bacteroidetes							
Bacteroidetes (B1)	130	0.14	-0.13	0.78	-0.02	0.66	-0.04
Bifidobacteria							
Bifidobacteria (A1b)	132	0.67	0.04	0.55	-0.05	0.24	-0.10
Other bacteria							
Enterobacteriacae (P1)	57	0.16	-0.19	*0.03**	*-0.28*	0.61	-0.07
M.smithii (E1)	25	*0.04**	*-0.42*	0.52	-0.13	1	0
A.muciniphila (V1)	127	0.79	-0.02	0.82	-0.02	0.78	-0.03

N=Number of individuals, P=P value of Spearman correlation, R=Spearman correlation coefficient

Figure 7: Temporal changes in abundance of bacterial groups and correlated changes in zBMI

Spearman regression analysis of the relative differences in zBMI from 9 to 18 months, 18 to 36 months, and 9 to 36 months with corresponding bacteria fold changes. Only the phylum level targets and bacteria giving significant correlations(*) are shown. Positive correlation between ΔzBMI and fold change increase of F1b, F6 and F8 between 9 and 18 months suggest that, *C.leptum* and *E.hallii* ,both converters of indigestible dietary carbohydrates (polysaccharides) to monosaccharide's and short-chain fatty acids (SCFA) may be specifically relevant, considering the correlation of overweight in this life stage with adult obesity

Conclusions

We found significant developments in the gut microbiota from 9 to 18 months, where cessation of breastfeeding and introduction of a Westernized diet induces replacement of a simpler, less diverse lactobacillus and bifidobacteria dominated microbiota with larger Clostridia (with polysaccharide preference) and Bacteroides (with animal fats, protein preference) targets. Moreover, we report the earliest signs of enterotype segregation as the development of microbiota characterized by either high or low relative levels of Bacteroides/Prevotella, seems to take place between 9 and 36 months. Correlations between ΔBMI and specific Clostridia from 9 to 18 months and ΔBMI and a tendency to a shift to the Prevotella-rich enterotype from 18 to 36 months may indicate specific carbohydrates to be of interest in relation to obesity development.

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